

## Negative regulation of FcεRI signaling by FcγRII costimulation in human blood basophils

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### Abstract:

**Background:** Signaling through the antigen receptors of human B and T cells and the high-affinity IgE receptor FcεRI of rodent mast cells is decreased by cross-linking these receptors to the low-affinity IgG receptor FcγRII. The inhibition is thought to involve the tyrosine phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the FcγRIIB cytoplasmic tail, creating binding sites for SH2-containing protein (Src homology domain containing protein tyrosine phosphatase 1 and 2 [SHP-1, SHP-2]) and/or lipid (SH2 domain-containing polyphosphatidyl-inositol 5-phosphatase) phosphatases that oppose activating signals from the costimulated antigen receptors. **Objective:** In human basophils and mast cells FcεRI signaling generates mediators and cytokines responsible for allergic inflammation. We proposed to determine whether FcεRI signaling is inhibited by FcγRII costimulation in human basophils and to explore the underlying mechanism as an approach to improving the treatment of allergic inflammation. **Methods:** FcγR expression on human basophils was examined using flow cytometry and RT-PCR analysis. FcγRII/FcεRI costimulation was typically accomplished by priming cells with anti-dinitrophenol (DNP) IgE and anti-DNP IgG and stimulating with DNP-BSA. Phosphatases were identified by Western blotting, and their partitioning between membrane and cytosol was determined by cell fractionation. Biotinylated synthetic peptides and phosphopeptides corresponding to the FcγRIIB ITIM sequence were used for adsorption assays. **Results:** We report that peripheral blood basophils express FcγRII (in both the ITIM-containing FcγRIIB and the immunoreceptor tyrosine-based activation motif-containing FcγRIIA forms) and that costimulating FcγRII and FcεRI inhibits basophil FcεRI-mediated histamine release, IL-4 production, and Ca<sup>2+</sup> mobilization. The inhibition of basophil FcεRI signaling by FcγRII/FcεRI costimulation is linked to a significant decrease in Syk tyrosine phosphorylation. Human basophils express all 3 SH2-containing phosphatases. **Conclusions:** Evidence that FcγRII/FcεRI costimulation induces SHP-1 translocation from the cytosolic to membrane fractions of basophils and that biotinylated synthetic peptides corresponding to the phosphorylated FcγRIIB ITIM sequence specifically recruit SHP-1 from basophil lysates

particularly implicates this protein phosphatase in the negative regulation of FcεRI signaling by costimulated FcγRII.

**Keywords:** Human | mast cell | basophil | signal transduction | Fc receptors | allergy | FcεRI

## Article:

### Abbreviations

BCR: B-cell antigen receptor

DNP: Dinitrophenol

ITAM: Immunoreceptor tyrosine-based activation motif

ITIM: Immunoreceptor tyrosine-based inhibitory motif

PMSF: Phenylmethylsulfonyl fluoride

PY: Phosphotyrosine

SHIP: SH2-containing inositol phospholipid 5-phosphatase

SHP-1: Src homology domain containing protein tyrosine phosphatase 1

SHP-2: Src homology domain containing protein tyrosine phosphatase 2

TCR: T-cell antigen receptor

Signaling through members of the multichain immune recognition receptor family, which includes the B-cell antigen receptor (BCR), the T-cell antigen receptor (TCR), the high-affinity IgE receptor (FcεR1), and the FcγR1 and FcγRIII classes of IgG receptors, is mediated by immunoreceptor tyrosine-based activation motifs (ITAMs) within receptor subunit cytoplasmic tails. ITAMs are 26- to 27-amino acid sequences consisting of dual YXXL sequences separated by approximately 10 amino acids.<sup>1</sup> Receptor activation leads to tyrosine phosphorylation of ITAMs by Src kinase family members, creating binding sites for the tandem SH2 domains of Syk kinase family members and initiating downstream signaling events. Recently, a motif transmitting negative signals, the immunoreceptor tyrosine-based inhibitory motif (ITIM), was identified first in FcγRIIB and subsequently in a series of immune system receptors. ITIMs are generally defined as 13-amino acid sequences including a consensus I/VxYxxL sequence.<sup>2</sup> ITIM-containing receptors can be grouped into two major classes on the basis of extracellular domains that identify them as members of the Ig superfamily (eg, FcγRIIB isoforms, KIR, gp49B1, CD22, PIR-B, and ILT-2) or as C-type lectins (eg, Ly-49 and MAFA). The inhibitory function of ITIMs is generally attributed to the ability of phospho-ITIMs to recruit SH2 domain-containing phosphatases, particularly the protein tyrosine phosphatases SHP-1 and SHP-2, and the SH2 domain-containing polyphosphatidylinositol 5-phosphatase (SHIP).<sup>3</sup>

To date, the negative regulation of signaling through ITAM-containing receptors by signals from co-cross-linked ITIM-containing FcγRIIB isoforms has been demonstrated in mouse and human B cells that naturally coexpress BCR and FcγRIIB1 and in T-cell and mast cell lines that express TCR or FcεRI plus endogenous or transfected FcγRIIB1.<sup>4</sup> Negative regulation by other ITIM-containing receptors has been demonstrated in mouse and human B cells (inhibition of BCR signaling by co-cross-linked CD22 and PIR-B<sup>5</sup>), in human natural killer cells (inhibition of FcγRIII signaling by co-cross-linked KIR<sup>6</sup>), in mouse bone marrow-derived mast cells (inhibition of FcεRI signaling by co-cross-linked gp49<sup>7</sup>), and in the RBL-2H3 rat mast cell line

(inhibition of FcεRI signaling by a variety of co-cross-linked or coligated receptors, including endogenous MAFA<sup>8</sup> and CD81<sup>9</sup> and transfected PIR-B cytoplasmic domain<sup>10</sup> and ILT-2<sup>11</sup>).

Here we show that human basophils express FcγRII (CD32) in both the ITIM-containing FcγRIIB and the ITAM-containing FcγRIIA forms but not FcγRI (CD64) or FcγRIII (CD16); we demonstrate negative signaling between FcγRII and FcεRI in these cells; and we link this negative signaling to changes in Syk activation and phosphatase distribution.

## Methods

### Antibodies

Anti-FcεRI α subunit mAb 22E7 (IgG1)<sup>12</sup> was a gift from Dr J. Kochan (Hoffman-La Roche, Nutley, NJ). Affinity-purified rabbit polyclonal antiphosphotyrosine (anti-PY) antibodies were prepared as described previously.<sup>13</sup> Monoclonal anti-Lyn, anti-SHIP, anti-SHP-1, and anti-SHP-2 (SH-PTP1 and SH-PTP2) were from Santa Cruz Biotechnology (Santa Cruz, Calif). Nonspecific mouse IgG1 (MOPC31C), anti-CD16 (IgG1), and FITC anti-PY mAbs were from Sigma (St Louis, Mo). The anti-FcγRII mAb AT10 recognizing all FcγRII isoforms (anti-CD32; IgG1) was from Serotec Ltd (Oxford, England). FcγRII expression was also probed with mAb IV (IgG1; A gift from Dr Carolyn Mold, University of New Mexico) specific for the FcγRIIA isoform.<sup>14</sup> Anti-FcγRI (anti-CD64; IgG1) antibodies were from Biosource (Camarillo, Calif). Monoclonal anti-Syk (IgG2a) was from Zymed Labs (San Francisco, Calif). Anti-F(ab')<sub>2</sub> FITC anti-mouse and anti-rabbit antibodies were from Jackson Labs (West Grove, Pa). Anti-4-hydroxyl-3-nitro-phenacetyl IgE (obtained from the cell line JW8 (ECACC #87080706) and NIP-(4-hydroxyl-3-nitro-5-iodo-phenylacetyl) were gifts from Graham Mackay (University of New Mexico).

Antidinitrophenol (anti-DNP) IgE mAb was prepared as described previously.<sup>15</sup> Hybridomas secreting anti-DNP IgG mAbs were obtained by the fusion of spleen cells of a BALB/c mouse immunized subcutaneously 6 times with DNP<sub>26</sub>-ovalbumin (10 μg per immunization emulsified with Freund's adjuvant) with mouse myeloma cells. Four weeks after the last immunization, a final boost of 10 μg of the same antigen (without adjuvant) was given intraperitoneally. Three days later, spleen cells were fused with SP2/0-Ag14 mouse myeloma cells by using polyethyleneglycol.<sup>16</sup> Hybridomas secreting DNP-specific antibodies were selected by means of ELISA assays with plates that had been coated with bovine albumin and derivatized in situ with 2,4,6-trinitrobenzene sulfonic acid in borate buffer. After incubating culture supernatants for 2 hours at 37°C, binding was revealed by using anti-mouse IgG coupled to alkaline phosphatase (Zymed Labs). Selected hybridomas were cloned by means of limiting dilution and repeat ELISA. Isotypes were determined with a commercial kit (Mouse Monoclonal Antibody Isotyping Kit, Sigma). Cloned hybrids were grown as ascitic fluid in the peritoneal cavity of BALB/c mice injected 10 days earlier with Pristane (Sigma). The antibodies were purified from clarified ascitic fluid by using affinity chromatography on columns with DNP-BSA coupled to cyanogen bromide-activated Sepharose 4B. Antibodies were eluted with 0.1 mol/L 2,4-DNP and passed through a Dowex 1X8-400 column, and fractions containing antibodies (detected by UV absorbance) were pooled and tested by means of flow cytometry for binding to acid-stripped basophils.

## Isolation of basophils

Venous blood (50-150 mL) was collected from normal donors after informed consent was obtained. Basophils were enriched to 15% to 65% purity by using Percoll gradient centrifugation.<sup>17</sup> Contaminating cells were removed by negative selection with an mAb cocktail for human basophil enrichment (StemCell Technologies, Vancouver, British Columbia, Canada) and magnetic bead separation (Miltenyi Biotec, Auburn, Calif).<sup>18</sup> In some experiments negatively selected basophils (>90% pure) were flow sorted (95 to >99.9% purity) on the basis of forward- and side-scatter characteristics.<sup>17</sup>

## Fcγ receptor analysis by flow cytometry

To detect FcγR classes, Percoll-enriched basophils were incubated for 30 minutes at 4°C in PBS-BSA with 10 μg/mL mouse mAbs to human FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) and then washed and incubated for another 30 minutes at 4°C with a 1:200 dilution of F(ab')<sub>2</sub> FITC goat anti-mouse antibodies. To identify mouse anti-DNP mAbs that bound in a stable fashion to basophil FcγRII, acid-stripped basophils<sup>19</sup> were incubated with 5 μg/mL DNP-specific mAbs for 1 to 3 hours at 4°C and then washed and incubated for 30 minutes at 4°C with a 1:200 dilution of F(ab')<sub>2</sub> FITC goat anti-rabbit antibodies. Washed cells were resuspended in 400 μL of PBS-BSA for flow cytometry. Basophils were identified on the basis of their forward- and side-scatter pattern, as described previously,<sup>17</sup> and mean channel fluorescence was determined for at least 10,000 cells by using a Coulter Epics Elite flow cytometer. MOPC31C nonspecific mouse IgG1 or nonimmune rabbit IgG were used as negative controls.

## Fcγ receptor analysis by RT-PCR and Southern blotting

Total cellular RNA was extracted using the Micro-FastTrack Method (Invitrogen, San Diego, Calif) from  $4 \times 10^5$  positively selected flow-sorted basophils and from the monocyte-lymphocyte cell population (PBL-no basophils) obtained after Percoll gradient centrifugation. RNA was concentrated by ethanol precipitation, resuspended in diethylpyrocarbonate-treated water, and stored at -70°C. Complementary (c)DNA was synthesized from 20 μL of RNA by using a first-strand cDNA kit (Pharmacia Biotech, Piscataway, NJ). FcγRII isoforms were specifically amplified by using 3 sets of primers: STB and C2-ac (IIA-specific); STC and C2-b (IIB-specific); and STC and C2-ac (IIC-specific). STC has been previously described.<sup>20</sup> New primers included the following: 5'-CCAACTTTGTCAGCCTCAT-3'(C2-b), a 3'-end primer located in the C2 exon specific for the FcγRIIB isoform; 5'-TCAAAT-GGGCAGCCTTCA-3' (C2-ac), a 3'-end primer located in the C2 exon specific for both FcγRIIA and FcγRIIC isoforms; and 5'-CAGAATGTATGTCCCAGAA-3' (STB), a 5'-end primer for the start of the IIA isoform. Actin primers were as follows: 5'-TTCTACAATGAGCTGCGTGTG and 3'-CACTGTGTTGGCATAGAGGTC. PCR amplification conditions consisted of one cycle with a denaturing step at 94°C for 2 minutes, annealing at 52°C for 2 minutes, and an extension at 72°C for 5 minutes followed by 40 cycles of a denaturing step at 94°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 1 minute. The last cycle was followed by a 15-minute extension step at 72°C. Southern blot analysis of PCR products was performed by using a

digoxigenin-uridine 5'-triphosphate (Boehringer Mannheim, Indianapolis, Ind) RS91-46 probe, as previously described.<sup>20</sup>

### Cell activation

Untreated or acid-stripped Percoll-enriched basophils were suspended in fresh Iscove's medium (Iscove's modified Dulbecco's medium containing 5% heat-inactivated FBS, nonessential amino acids, and 5 µg/mL gentamicin) and primed in 5-mL tubes with 10 µg/mL mouse anti-DNP IgE (or anti-NP-IgE), mouse anti-DNP IgG, or a combination of both for 2 hours at 37°C in a 5% CO<sub>2</sub> incubator. Cells were washed with HBSS<sup>-</sup> (HBSS without Ca<sup>2+</sup> or Mg<sup>2+</sup>),<sup>18</sup> resuspended to 0.5 to 1.1 × 10<sup>6</sup> basophils/mL in prewarmed HBSS<sup>+</sup> (HBSS with 1.4 mmol/L CaCl<sub>2</sub> and 1 mmol/L MgCl<sub>2</sub>) with DNP-BSA (0.005-0.01 µg/mL) or NIP-BSA (0.001-1 µg/mL), and activated by incubation at 37°C for 45 minutes (his-tamine) or 4 hours (IL-4) at 37°C in a 5% CO<sub>2</sub> incubator. Reactions were stopped by centrifugation at 150g at 4°C. Supernatants were stored at -70°C. Histamine release was measured as previously described.<sup>18</sup> IL-4 was measured in the supernatants by using a kit from Biosource.

### Ca<sup>2+</sup> mobilization

Percoll-enriched negatively selected basophils were transferred to poly-L -lysine-coated glass coverslips and loaded with 2 µmol/L Fura-2AM (Sigma), as previously described,<sup>21</sup> during the last 30 minutes of priming with anti-DNP IgE and anti-DNP-IgG. Washed coverslips were transferred to a temperature-controlled dish with a 5% CO<sub>2</sub> atmosphere and mounted on the stage of an inverted Zeiss fluorescence microscope. The microscope is equipped with a 100-W Hg lamp and computer-controlled filter wheels and shutters that allow excitation light to pass alternately through 10-nm bandpass filters centered at 360 and 385 nm. Emitted fluorescence was collected at 510 nm by using a Sony CCD camera interfaced to a COMPIX image analysis station. After acquisition of baseline fluorescence values for approximately 2 minutes, DNP-BSA was added by pipette, and fluorescence intensities collected for a further 6 to 8 minutes. Data were corrected for background, and average ratio values for each cell in a field were calculated for user-defined areas within each cell. Average ratio values were converted to [Ca<sup>2+</sup>]<sub>i</sub> on the basis of calibration solutions containing maximal and minimal Ca<sup>2+</sup> concentrations.<sup>21</sup> Each experiment provided time-resolved analysis of Ca<sup>2+</sup> levels for 4 to 15 individual cells within a single field of view.

### Lyn and Syk phosphorylation

Negatively selected flow-sorted basophils (≥93% pure) were acid stripped and primed as above. After 2 minutes of activation, cells were lysed in 400 µL of 50 mmol/L TRIS (pH 7.5), 150 mmol/L NaCl, 1% Brij, 1 mmol/L sodium orthovanadate, and 1 µg/mL each of antipain, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride (PMSF; lysis buffer) and incubated for 10 minutes on ice. Immune complexes were generated by incubating clarified supernatants with anti-Lyn or anti-Syk preadsorbed to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden), as previously described.<sup>22</sup> The proteins were separated by SDS-PAGE and transferred to nitrocellulose sheets, and phosphoproteins were identified by using anti-PY immunoblotting with enhanced chemiluminescence detection reagents (ECL, Amersham Corp), as previously

described.<sup>18</sup> After probing with anti-PY antibody, blots were stripped for 30 minutes with 100 mmol/L 2-mercaptoethanol, 2% SDS (wt/vol), and 0.5 mol/L TRIS-HCl (pH 6.8) at 50°C and reprobed with antikinase antibody (1 µg/mL).

### Identifying phosphatases

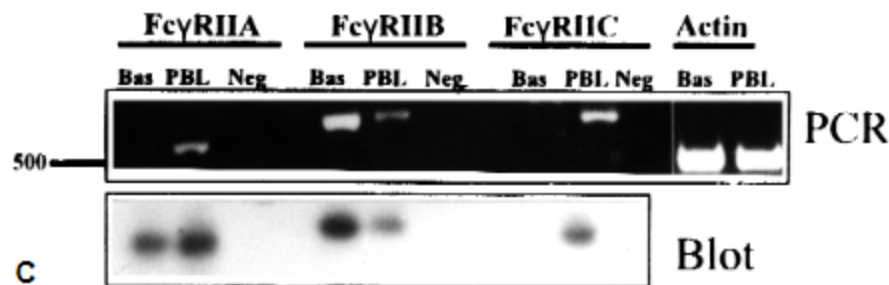
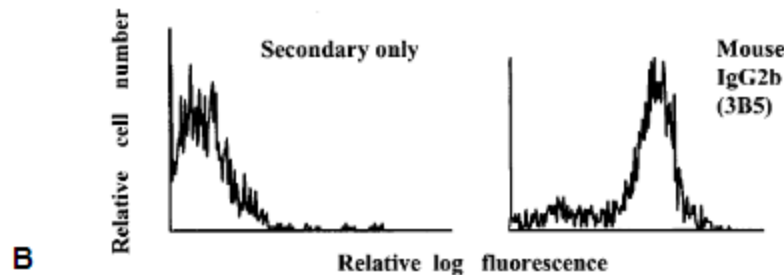
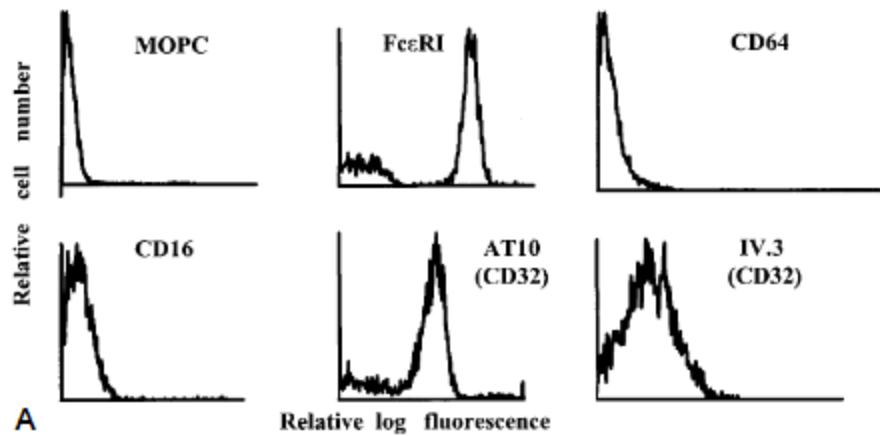
Negatively selected flow-sorted basophils ( $2-5 \times 10^5$  cells/condition), KU812 human basophil-like cells (ATCC), and HMC-1 human mast cell-like cells (generously provided by J. H. Butterfield, Mayo Medical School) were lysed by means of incubation on ice for 10 minutes in 0.1 mL of lysis buffer, and insoluble material was removed by centrifugation at 10,000g for 5 minutes. Proteins were separated by 10% PAGE by using  $1 \times 10^5$  cell equivalents per lane or 10 to 20 µg/lane and transferred to nitrocellulose, and phosphatases were identified by using Western blotting with antibodies to either SHP-1, SHP-2, or SHIP (1 µg/mL) at 4°C in PBS-BSA followed by enhanced chemiluminescence detection.

### Partitioning of SHP-1 between cytosol and membranes

Negatively selected basophils ( $2-5 \times 10^5$  cells/condition) were suspended at  $3 \times 10^7$  cells/mL in hypotonic lysis buffer (20 mmol/L TRIS [pH 7.5], 5 mmol/L EDTA, 5 mmol/L ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 5 mmol/L dithiothreitol, 1 mmol/L PMSF, and 100 µg/mL each of leupeptin, aprotinin, and antipain).<sup>23</sup> The cells were lysed by placing the tubes at -70°C for 15 minutes and then thawing to 4°C. Debris and nuclei were removed by centrifugation at 500g at 4°C. The remaining supernatants were centrifuged at 100,000g for 10 minutes at 4°C. The supernatant (cytosolic) and pellet (membrane) fractions from high-speed centrifugations were resuspended directly in 2× or 1× lysis buffer, respectively, and analyzed for the presence of SHP-1, SHP-2, and SHIP by using SDS-PAGE and Western blotting, as described above. The ECL films from these experiments were scanned and converted to digital format. The translocation of SHP-1 was calculated as the percentage found in the cytosol or membrane fraction for each cross-linking condition divided by the sum of the cytosol and membrane fraction from unstimulated cells.

### Cell lysate adsorption to ITIM peptides

Negatively selected basophils ( $1.5-2.0 \times 10^5$  cells/condition) were lysed in 250 µL of NP-40 lysis buffer (150 mmol/L NaCl, 10 mmol/L TRIS, 1% Nonidet P-40, 10 mmol/L Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/L PMSF, 5 mmol/L NaF, 0.4 mmol/L EDTA, and 10 µg/mL each of leupeptin, aprotinin, and antipain). Portions (100 µL) of the clarified lysate were incubated for 30 minutes at 4°C with 100 µL of agarose-immobilized streptavidin (Pierce, Milwaukee, Wis) plus 5 µg of synthetic biotinylated nonphosphorylated or tyrosine-phosphorylated peptides with the FcγRIIB ITIM sequence KGGGAENTIT(p)YSLLKH (Macromolecular Resources, Fort Collins, Colo). After the incubation, the lysate-bead mixtures were centrifuged at 10,000g for 1 minute, and the beads were resuspended in 2× sample buffer and analyzed with SDS-PAGE and Western blotting.



**Fig. 1.** **A**, Membrane expression of Fc $\gamma$ RII on human basophils. Percoll-enriched basophils (55% purity) were incubated with mouse antibodies to CD16 (Fc $\gamma$ RIII), CD32 (Fc $\gamma$ RII), or CD64 (Fc $\gamma$ RI) followed by FITC-labeled goat anti-mouse IgG antibodies and flow cytometry. An irrelevant IgG1 was substituted for the CD antibodies as a negative control. Monoclonal antibody 22E7, which binds the Fc $\epsilon$ RI  $\alpha$  chain, was the positive control. **B**, Human basophils bind mouse anti-DNP IgG. Acid-stripped basophils (43% purity) were incubated with a series of DNP-specific mouse mAbs followed by FITC-conjugated anti-mouse antibodies and flow cytometry. Monoclonal antibody 3B5 (IgG2b), but not two other DNP-specific antibodies (both IgG1; not shown), showed consistent binding to basophil Fc $\gamma$ RII. **C**, Expression of Fc $\gamma$ RII transcripts in human basophils. *Top*, RNA from highly purified basophils (99.9% pure) or peripheral blood leukocytes (*PBL*) was reverse transcribed, and Fc $\gamma$ RII transcripts were selectively amplified. Products were separated on 2% agarose gels and detected by using ethidium bromide staining. Actin-specific primers were used as positive controls, and reaction mixtures with primers only (no RNA) were used as negative controls. Fc $\gamma$ RIIA- and Fc $\gamma$ RIIB-specific primers amplified products of 796 and 754 bp, respectively, whereas actin primers amplified a product of 627 bp. *Bottom*, Southern blotting performed with a digoxigenin-UTP-specific probe recognizing all Fc $\gamma$ RII isoforms confirmed the PCR data. The results are representative of studies with two separate donors.

## Statistical analysis

Statistical analysis was performed by using Graphpad Prism software (San Diego, Calif). Data sets were tested for significant differences by using the paired *t* test. A *P* value of less than .05 was considered to be statistically significant.

## Results

### Identification of FcγRs on human basophils

The results in Fig 1, *A*, show that human basophils react strongly with the pan anti-FcγRII mAb AT10, weakly with the anti-FcγRIIA-specific mAb IV.3, and not at all with antibodies to CD64 (FcγRI) or CD16 (FcγRIII).

The results in Fig 1, *B*, show a strong peak of binding of the DNP-specific IgG2b mAb 3B5 to acid-stripped human basophils. In contrast, several other anti-DNP mAb isotypes bound weakly and variably to basophils (not shown). 3B5 was selected to prime FcγRII for antigen-mediated cross-linking.

The results of RT-PCR analysis, illustrated in Fig 1, *C*, show that human basophils contain transcripts for the ITIM-containing FcγRII isoform FcγRIIB and for the ITAM-containing FcγRII isoform FcγRIIA. No transcripts for FcγRIIC were detected in these cell preparations. The intensity of the basophil FcγRIIB band suggests that it is the predominant isoform expressed on human basophils.

### FcγRII/FcεRI costimulation inhibits FcεRI-dependent human basophil degranulation

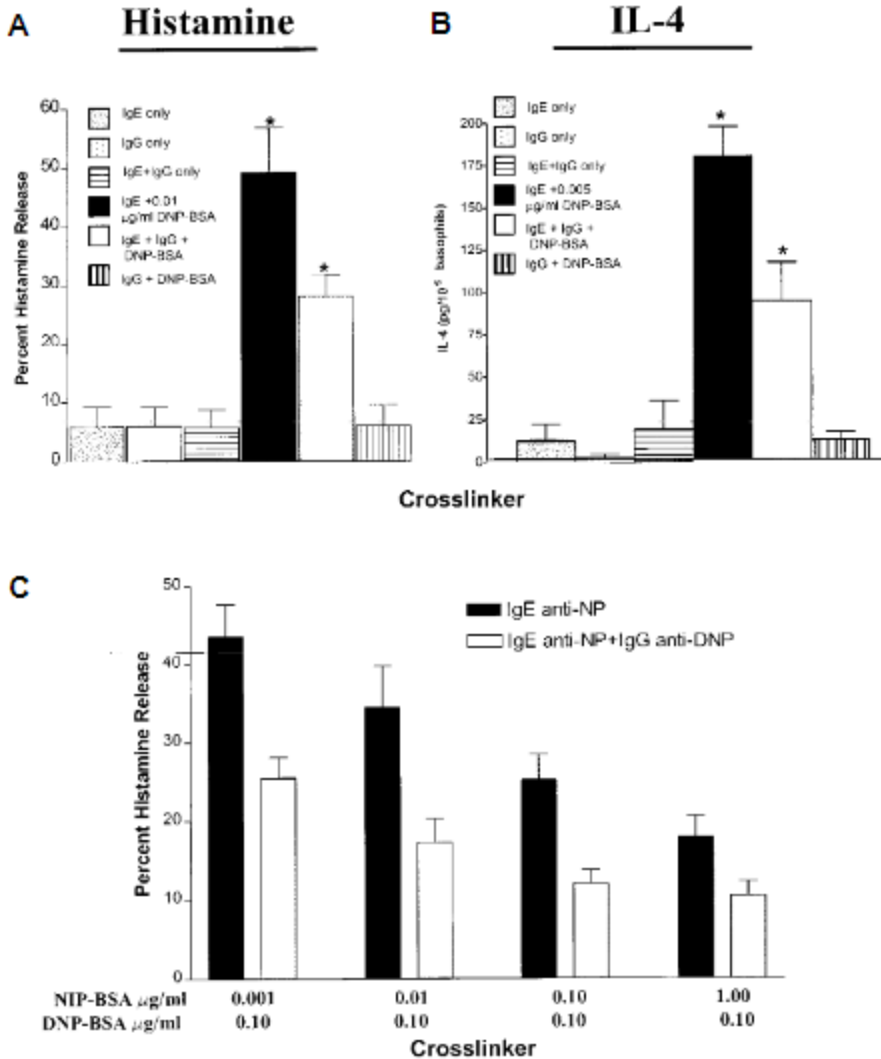
Fig 2, *A*, shows that no secretion is induced by simply priming Percoll-enriched basophils with DNP-specific IgE or IgG or by cross-linking anti-DNP IgG-FcγRII complexes with polyvalent DNP-BSA.

Cross-linking anti-DNP IgE-FcεRI complexes with DNP-BSA induces histamine release. In 5 separate experiments costimulating anti-DNP IgE-primed FcεRI and anti-DNP IgE-primed FcγRII with DNP-BSA reduced FcεRI-mediated secretion by an average of 43% ( $\pm$  7%).

### FcγRII/FcεRI costimulation inhibits FcεRI-mediated IL-4 production

Cross-linking anti-DNP IgE-FcεRI complexes with DNP-BSA for 4 hours induces IL-4 production and release (Fig 2, *B*). In 3 experiments costimulating anti-DNP IgE-primed FcεRI and anti-DNP IgE-primed FcγRII with DNP-BSA reduced FcεRI-mediated IL-4 release by an average of 36% ( $\pm$  12%).



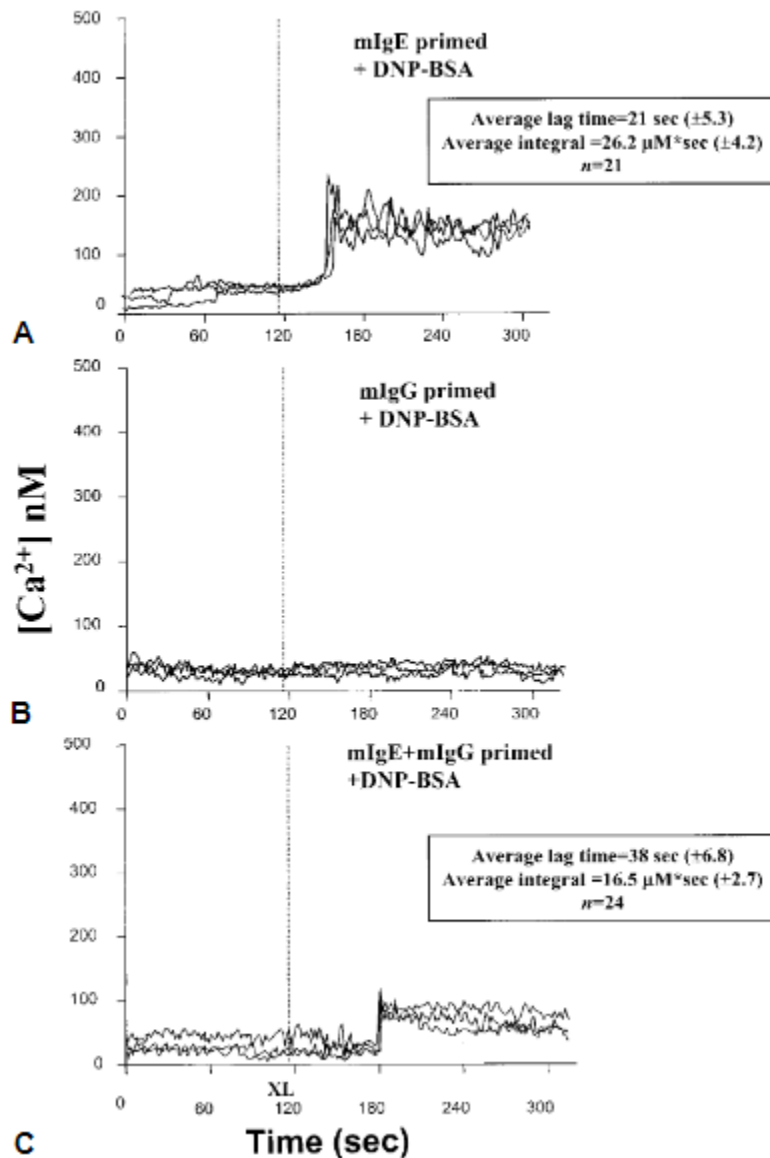


**Fig. 2.** Fc $\gamma$ RII/Fc $\epsilon$ RI costimulation inhibits Fc $\epsilon$ RI-mediated basophil degranulation and IL-4 production. **A**, Percoll-enriched acid-stripped basophils (20%-35% purity) were incubated with anti-DNP IgE (10  $\mu$ g/mL), anti-DNP IgG2b (10  $\mu$ g/mL), or both antibodies (priming). Histamine release into the medium was measured after stimulating the cells with 0.01  $\mu$ g/mL DNP-BSA for 45 minutes. Results are the average of 5 separate experiments ( $\pm$  SEM). **B**, Percoll-enriched acid-stripped basophils (15%-51% purity) were primed as in **A** and incubated with or without 0.005  $\mu$ g/mL DNP-BSA for 4 hours, and supernatants were assayed for IL-4 release. Results are the average of 3 separate experiments ( $\pm$  SEM). *Asterisks* represent values significantly reduced (**A**,  $P = .012$ ; **B**,  $P = .029$ ) when comparing cells primed with IgE alone with cells primed with IgE plus IgG. **C**, Inhibition of histamine release by costimulation is not due to competition for antigen. Percoll-enriched acid-stripped basophils (15%-25% purity) were incubated for 2 hours with anti-NP IgE (10  $\mu$ g/mL), anti-DNP IgG2b (10  $\mu$ g/mL), or with both antibodies. Histamine release was measured after stimulating the cells with mixtures of NIP-BSA and DNP-BSA for 45 minutes. Results are the average of two separate experiments ( $\pm$  SEM).

Fc $\gamma$ RII/Fc $\epsilon$ RI costimulation does not reduce signaling by lowering the availability of antigen to Fc $\epsilon$ RI

We considered the possibility that costimulation reduces Fc $\epsilon$ RI signaling as the result of competition between two anti-DNP-primed receptors for the same antigen. The results in Fig 2, *C*, show that this is probably not the case. Fc $\epsilon$ RI and Fc $\gamma$ RII were primed respectively with

antibodies specific for NP (using anti-NP IgE) or DNP (using anti-DNP IgG). Cells were then challenged alone or with combinations of the two antigens. DNP-BSA alone caused no secretion (data not shown). NIP-BSA alone induced secretion from anti-NP IgE-primed cells. In dually primed cells, adding DNP-BSA to stimulate Fc $\gamma$ R2 reduced the amount of Fc $\epsilon$ R1-mediated secretion that could be induced by NIP-BSA by up to 50%. Thus the inhibition of Fc $\epsilon$ R1 signaling by Fc $\gamma$ R2/Fc $\epsilon$ R1 costimulation occurs whether the two receptors are cross-linked by the same or different antigens.

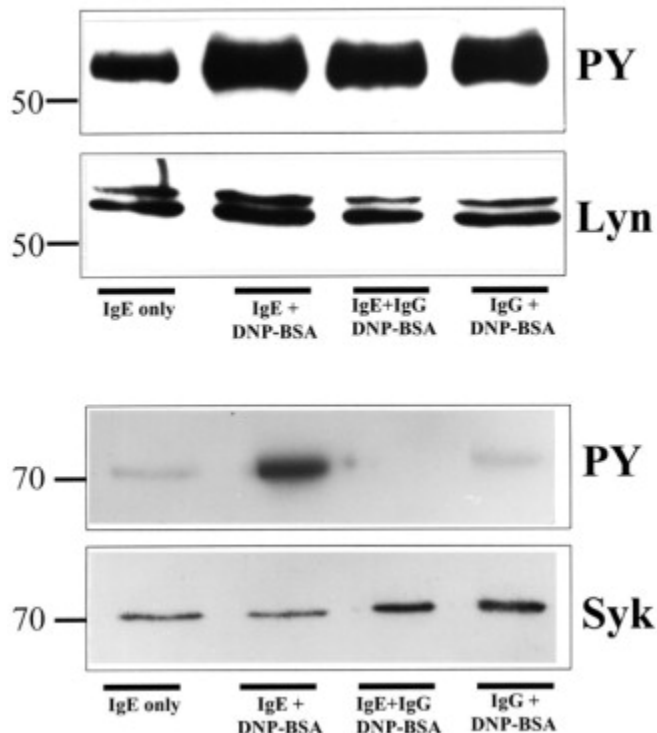


**Fig. 3.** Fc $\gamma$ R2/Fc $\epsilon$ R1 costimulation inhibits Fc $\epsilon$ R1-mediated Ca<sup>2+</sup> influx in human basophils. Percoll-enriched acid-stripped basophils (61% purity) were placed on coverslips and primed as in Fig 2. After fura-2 loading, the cells were placed on the microscope stage and challenged with 0.01  $\mu\text{g}/\text{mL}$  DNP-BSA. Each plot represents the Ca<sup>2+</sup> response of 3 representative cells. The total number of cells examined in 3 separate experiments and the SD of both the lag time to response and the integral of the response over 200 seconds after the initial antigen-stimulated increase in [Ca<sup>2+</sup>]<sub>i</sub> are noted for two of the 3 conditions. Differences were significant at a  $P$  value of less than .01 (lag time) and a  $P$  value of less than .001 (average integral), determined by using the unpaired  $t$  test with Welch's correction.

### FcγRII/FcεRI costimulation reduces FcεRI-mediated Ca<sup>2+</sup> mobilization

Fluorescence ratio imaging microscopy was used to measure the effects of FcγRII and FcεRI costimulation on intracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>). Cross-linking anti-DNP IgE-primed FcεRI with DNP-BSA leads, after a lag, to a rapid increase in intracellular Ca<sup>2+</sup> levels (Fig 3, *A*).

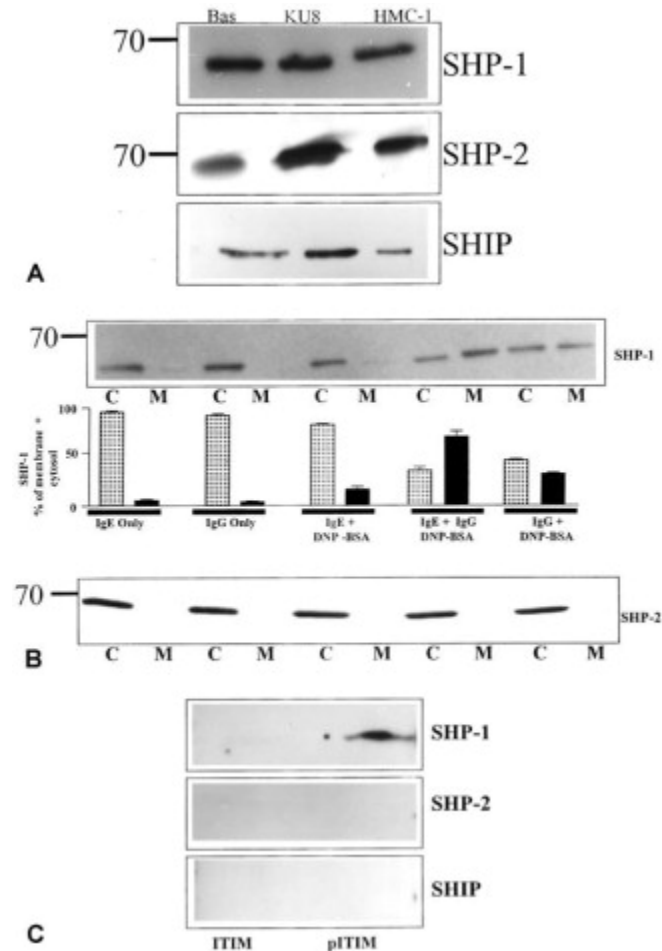
The Ca<sup>2+</sup> spike is followed by a sustained elevation in Ca<sup>2+</sup> levels (ie, the Ca<sup>2+</sup> plateau). In the 3 experiments reported here, the lag from antigen addition to anti-DNP IgE-primed cells to FcεRI-mediated Ca<sup>2+</sup> influx was around 21 seconds. The magnitude of the response, estimated by integrating Ca<sup>2+</sup> concentrations over 200 seconds after the initial Ca<sup>2+</sup> spike,<sup>21</sup> was around 26 μmol/L per second. No increase in intracellular Ca<sup>2+</sup> concentrations was detected in response to antigen in cells primed with anti-DNP IgG alone (Fig 3, *B*). When cells were primed with both DNP-specific IgE and IgG before antigen challenge, the lag time from antigen addition to the initial rise in [Ca<sup>2+</sup>] was approximately doubled, and the integrated Ca<sup>2+</sup> response was markedly diminished (Fig 3, *C*).



**Fig. 4.** FcγRII/FcεRI costimulation inhibits FcεRI-mediated Syk phosphorylation. Percoll-enriched negatively selected basophils (93% purity) were acid stripped, primed as in Fig 2, and challenged for 2 minutes with 0.01 μg/mL DNP-BSA. Cells were lysed, and Lyn (**A**) or Syk (**B**) was immunoprecipitated from the clarified lysates. In these experiments immunoprecipitates from  $4.3$  to  $9.4 \times 10^5$  basophils were analyzed by Western blotting with anti-phosphotyrosine antibodies (*PY*), followed by anti-Syk or anti-Lyn antibodies. The results are representative of two separate experiments.

### FcγRII/FcεRI costimulation inhibits FcεRI-mediated Syk phosphorylation

Lyn and Syk were immunoprecipitated from lysates of variously activated cells, and the immune complexes were resolved by means of SDS-PAGE and probed with antiphosphotyrosine followed by antikinase antibodies. As shown in Fig 4, Lyn, but not Syk, is tyrosine phosphorylated in resting basophils.



**Fig. 5. A,** Human basophils express SHP-1, SHP-2, and SHIP. Negatively selected flow-sorted basophils (>95% purity), KU812 cells, or HMC-1 cells were lysed and proteins (13  $\mu$ g/lane) were separated by using SDS-PAGE and Western blotted with anti-SHP-1, anti-SHP-2, or anti-SHIP antibodies. Results shown are representative of two separate experiments. SHIP was calculated to be between 140 and 145 kd. **B,** SHP-1 is translocated to the membrane after Fc $\gamma$ R2 cross-linking. Highly purified basophils (97% purity;  $2-5 \times 10^5$  cells/condition) were primed as in Fig 2 and challenged for 2 minutes with 0.01  $\mu$ g/mL DNP-BSA. Membrane and cytosolic fractions were separated by SDS-PAGE and immunoblotted with anti-SHP-1 or anti-SHP-2 antibodies. Results shown are representative of 5 (SHP-1) or 3 (SHP-2) separate experiments. The *bottom panel* shows the amount of SHP-1 in the cytosol or membrane expressed as a percentage of the sum of SHP-1 in resting cells. **C,** The Fc $\gamma$ R2B phospho-ITIM peptide selects SHP-1 from human basophil lysates. Lysates of negatively selected flow-sorted basophils (96% purity) were incubated with biotinylated nonphosphorylated or tyrosine-phosphorylated Fc $\gamma$ R2B ITIMs plus agarose-immobilized streptavidin. After 30 minutes, the beads were washed and analyzed by SDS-PAGE and Western blotting. Whole cell lysates were used as a positive control for each experiment (data not shown).

As reported previously,<sup>18</sup> cross-linking anti-DNP IgE-primed Fc $\epsilon$ RI with DNP-BSA induces a substantial tyrosine phosphorylation of Lyn and Syk. The amount of Syk tyrosine phosphorylation is markedly reduced in IgE plus IgG-primed cells, when DNP-BSA stimulates

both Fc $\gamma$ RII and Fc $\epsilon$ RI. Lyn phosphorylation appeared to be very similar between IgE-primed, IgG-primed, or dually primed and activated cells (Fig 5, *B* ).

#### Human basophils express SHP-1, SHP-2, and SHIP

Lysates of human basophils were probed for the protein tyrosine phosphatases SHP-1 and SHP-2 and the polyphosphoinositide 5-phosphatase SHIP. As shown in Fig 5, *A* , SHP-1 and SHP-2 were both readily detected in human basophils. SHIP was also present but required longer exposure time to the ECL reagents for detection.

#### Fc $\gamma$ RII cross-linking induces the translocation of SHP-1 from cytosol to membranes

The results in Fig 5, *B* , show that SHP-1 is mainly in the cytosolic fraction of IgE- and IgG-primed human basophils. Cross-linking Fc $\epsilon$ R1 on anti-DNP IgE-primed cells with DNP-BSA does not affect the partitioning of SHP-1 between cytosol and membranes. In contrast, a substantial proportion of SHP-1 is membrane associated when Fc $\gamma$ RII and Fc $\epsilon$ R1 are costimulated by the addition of DNP-BSA to cells sensitized with anti-DNP IgE plus anti-DNP IgG. Remarkably, simply cross-linking anti-DNP IgG-primed Fc $\gamma$ RII with DNP-BSA also recruits SHP-1 from the cytosol to the membrane. SHP-2 remained in the cytosolic fraction under all conditions. In these experiments SHIP was not detected in either cytosol or membrane fractions, very likely reflecting excess dilution of inherently scarce proteins during cell fractionation.

#### Fc $\gamma$ RIIB phospho-ITIM peptide selectively adsorb SHP-1 from human basophil lysates

Basophil lysates were incubated with biotinylated peptides corresponding to the murine Fc $\gamma$ RIIB sequence KTEAENTITYSLLK (highly homologous to the human sequence)<sup>24</sup> in both the phosphorylated and nonphosphorylated forms, and the peptides were recovered by incubation with streptavidin-Sepharose beads and analyzed by using SDS-PAGE. Fig 5, *C* , shows that the Fc $\gamma$ RIIB phospho-ITIM specifically adsorbs SHP-1 from basophil lysates. No SHP-1 is adsorbed to the nonphosphorylated Fc $\gamma$ RIIB ITIM.

### Discussion

The ability of the ITIM-containing IgG receptor Fc $\gamma$ RIIB to act as a negative regulator of ITAM-containing immunoreceptors was recognized initially through studies of the antigen receptor of B cells and was subsequently extended to studies of T-cell activation through the TCR.<sup>4</sup> Fc $\gamma$ RIIB was first implicated in the negative regulation of Fc $\epsilon$ R1-mediated signaling by Daeron et al.<sup>25</sup> These investigators sensitized acid-stripped basophils with murine IgE and activated them with anti-mouse Ig antibodies with or without preincubation with anti-CD32 mAb; Fc $\gamma$ RII and Fc $\epsilon$ R1 costimulation led to inhibition of Fc $\epsilon$ RI-induced histamine release. Daeron et al<sup>26</sup> extended their analysis of Fc $\gamma$ RII/Fc $\epsilon$ R1 crosstalk by developing stably transfected RBL-2H3 cells expressing murine and human Fc $\gamma$ RIIB isoforms. In the rat model system they confirmed that the 13-amino acid ITIM sequence in the human Fc $\gamma$ RIIB was necessary to inhibit Fc $\epsilon$ R1-mediated secretion and raised the possibility that allergen immunotherapy may work in part by promoting negative signaling from Fc $\gamma$ RIIB to Fc $\epsilon$ RI. These landmark studies left open several critical issues,

including the identity of FcγRII isoforms expressed on human basophils and evidence that co-cross-linking IgE and IgG directed at the same polyvalent antigen downregulates the FcεRI-mediated activation of human cells.

There are multiple FcγRII isoforms, representing the products of 3 distinct genes.<sup>4</sup> FcγRIIA occurs in both a transmembrane form with an ITAM and a soluble form that results from alternative splicing. Three FcγRIIB isoforms, all transmembrane proteins with cytoplasmic ITIMs, arise by means of alternative splicing. FcγRIIC is a single membrane-spanning receptor that also carries a cytoplasmic ITAM. Natural killer cells express 4 different transcripts of the gene encoding FcγRIIC.<sup>20</sup> Here we have confirmed that human basophils express CD32 (FcγRII), but not CD16 (FcγRIII) or CD64 (FcγRI).<sup>27, 28, 29</sup> Our PCR results establish that human basophils contain transcripts for both the ITIM-containing FcγRIIB and the ITAM-containing FcγRIIA.

We report that co-cross-linking of anti-DNP IgG-primed FcγRII and anti-DNP IgE-primed FcεRI on human basophils with specific antigen (DNP-BSA) inhibits FcεRI-induced degranulation and also inhibits FcεRI-mediated IL-4 production. These results provide the first demonstration of antigen-specific downregulation of FcεRI-mediated signaling through FcγRII/FcεRI costimulation on human basophils. FcεRI-induced degranulation is also inhibited when anti-DNP IgG-primed FcγRII and anti-NP IgE-primed FcεRI on human basophils are costimulated with separate antigens (DNP-BSA and NP-BSA). Thus negative signaling from FcγRII to FcεRI can occur when the receptors are co-stimulated with separate antigens, as well as when they are co-cross-linked with the same antigen. For this reason, although the majority of experiments reported here were performed under co-cross-linking conditions, we describe our experimental conditions as costimulation.

In RBL-2H3 cells and human basophils FcεRI-mediated degranulation and IL-4 production depend on the Ins(1,4,5)P<sub>3</sub>-dependent release of Ca<sup>2+</sup> from intracellular stores and on the subsequent sustained influx of Ca<sup>2+</sup> from the medium by both capacitative and receptor-coupled pathways.<sup>21, 30</sup> We resolved the two phases of the Ca<sup>2+</sup> mobilization response by using ratio imaging microscopy. FcγRII/FcεRI costimulation extends the lag time from FcεRI cross-linking to the Ca<sup>2+</sup> spike response, which reflects the release of Ca<sup>2+</sup> stores, and also reduces the magnitude of the Ca<sup>2+</sup> plateau, which is attributed principally to Ca<sup>2+</sup> influx. Previous studies examining FcγRII/BCR co-cross-linked B cells reported premature termination of Ca<sup>2+</sup> influx, which is sometimes associated with a reduced Ca<sup>2+</sup> spike response but not an increased delay to the spike response.<sup>31, 32, 33, 34</sup> The difference is unexplained but may simply reflect the poorer time resolution of population-averaged spectrofluorometric assays in comparison with single cell microscopic assays of Ca<sup>2+</sup> mobilization. Alternatively, the differences may reflect distinct signaling properties of B cells and basophils.

Phosphorylation of the tyrosine kinase Syk is a key event linking FcεRI cross-linking to downstream signaling pathways in rodent mast cells<sup>22, 35, 36</sup> and human basophils.<sup>18, 37</sup> Here we show a modest level of basal Lyn phosphorylation and little or no tyrosine-phosphorylated Syk in either resting cells or in cells where only FcγRII was cross-linked. FcεRI cross-linking resulted in increased Lyn and Syk phosphorylation. Syk, but not Lyn, phosphorylation was markedly decreased by co-stimulating FcγRII and FcεRI. The functional significance of

changing Lyn phosphorylation is difficult to interpret because this Src family kinase is phosphorylated on both inhibitory and stimulatory sites. On the other hand, Syk has no inhibitory tyrosine phosphorylation sites, and therefore its increased tyrosine phosphorylation is a strong indicator of its increased catalytic activity.<sup>4</sup> Our data differ from the results of Malbec et al,<sup>38</sup> who found in transfected RBL-2H3 cells that Syk phosphorylation was unaffected by FcγRII/FcεRI co-cross-linking. The results presented here resemble previous studies in human B cells showing reduced levels of Syk tyrosine phosphorylation when FcγRIIB was cross-linked to the BCR.<sup>31, 32</sup>

The results of Western blot analysis established that human basophils express all 3 phosphatases previously implicated in negative signaling: SHP-1, SHP-2, and SHIP. Further studies by cell fractionation showed that SHP-1 is translocated from the cytosolic to membrane fractions of basophils during FcεRI and FcγRII costimulation, as well as in response to FcγRII co-cross-linking alone. Fractionation studies do not identify the membrane-binding sites for SHP-1. However, synthetic biotinylated peptides corresponding to the phosphorylated FcγRIIB ITIM sequence recruit SHP-1 from basophil lysates. These data implicate phosphorylated FcγRIIB ITIM sequences as likely targets for membrane-bound SHP-1.

Several issues remain to be explored. First, published data link inhibitory signaling from FcγRII to the BCR or to the FcεRI of transfected RBL-2H3 cells principally to the interaction of SHIP with the phospho-ITIMs of FcγRIIB.<sup>38, 39, 40, 41, 42</sup> It remains possible that SHIP contributes importantly to the inhibition of FcεRI signaling by costimulating FcγRII in human basophils and that more sensitive detection methods will reveal its involvement. On the other hand, SHP-1 is strongly implicated in the inhibitory signaling that occurs from the ITIM-containing receptors KIR and CD22 to the BCR and from the ITIM-containing receptors gp49B and PIR-B to the FcεRI.<sup>33, 34, 35, 41, 43, 44, 45, 46</sup> Second, it is unclear whether the reduced phosphorylation of Syk caused by FcεRI/FcγRII costimulation results from the dephosphorylation of FcεRI phospho-ITAMs, which could reduce Syk recruitment and activation, or from the dephosphorylation of Syk itself. A direct association of Syk and SHP-1 has been demonstrated previously in B cells.<sup>47</sup> In addition to Syk,<sup>47, 48</sup> there is a growing list of receptor-associated tyrosine kinases that are inhibited by SHP-1,<sup>49, 50, 51</sup> raising the possibility that Syk may not be the only target of negative regulation in human basophils. Finally, we have not yet addressed the mechanism of SHP-1 recruitment induced by FcγRII cross-linking alone in human basophils. It is possible that FcγRII cross-linking on basophils may generate hybrid complexes (FcγRIIB plus FcγRIIA) in which the ITAM-containing FcγRIIA may recruit and activate tyrosine kinases that in turn phosphorylate FcγRIIB ITIMs, creating membrane-binding sites for SHP-1.

In summary, Syk phosphorylation and positive signaling through the high-affinity IgE receptor FcεRI on human basophils can be inhibited by costimulating the low-affinity IgG receptor FcγRII. We hypothesize that this negative signaling from FcγRII to FcεRI occurs at least in part by the recruitment of SHP-1 to the FcγRIIB ITIM, positioning it to block or reverse the phosphorylation and activation of Syk needed to couple FcεRI crosslinking to downstream responses.

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